Nanoparticles and Ocean Optics

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LONG-TERM GOALS

Our long-term goal is to understand the role of the two most abundant nanoparticles in the sea, viruses and polymer aggregates, in controlling the inherent ocean optical properties of seawater.

OBJECTIVES

There are two objectives to this work that deal with the role of nanoparticles and ocean optics:

1. To determine the importance of viruses (and other biological processes) in the formation of chromophoric dissolved organic matter (CDOM) in the ocean (including the time scales of infection/lysis and CDOM release)

2. To quantify the importance of aggregate formation to seawater optical properties as well as the potential for non-biogenic calcification associated with aggregate formation

APPROACH

The initial focus for the Virology portion of this project (start date January '05) was the acquisition and characterization of virus/host assemblages for use in initial lab-based dilution experiments. We tested methods for the separation of naturally occurring virus and host from seawater samples in preparation for dilution experiments with natural assemblages. Next, we examined the role of viruses in production of CDOM using a modified version of the Landry dilution method (Landry et al. 1995). Rather than using the dilution technique to focus on phytoplankton growth and mortality from grazing, we targeted CDOM production by all living cells, using UV absorption as the indicator of CDOM. We tested this dilution approach on heterotrophic bacterial hosts and their specific viruses, in a simplified experimental system, to see if we could see CDOM release from viral lysis and if we could

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control infection by the dilution process. Subsequent dilution experiments used eukaryotic hosts (*Emiliania huxleyi*) and its specific viruses.

In October of 2005, we will transition these experiments to the field, where a field sample containing a variety of hosts will be diluted). We will separate a natural sea water assemblage off the Bigelow Lab dock into a concentrated suspension of free viruses (<200nm), and a fraction with particles >200nm that contains mostly hosts (prokaryotes and eukaryotes). This will be accomplished using tangential flow ultrafiltration, which enhances the concentration of only particles <200nm (such as viruses) in one fraction and concentrates other larger grazers (like ciliates, heterotrophic flagellates, heterotrophic dinoflagellates, etc.) in the other. For the actual dilution experiment, we will recombine these fractions in dilution-style experiments at different concentrations (but with the same multiplicity of infection, MOI) and follow the release of CDOM as viruses come into closer contact with hosts. Instead of making diluent from 0.2μm-filtered seawater, we will make it with 0.02μm-filtered seawater, so this diluent will be virus-free. Ultra-filtration has been used before to sequester and concentrate free-floating viruses in natural assemblages so that one could examine associated effects on their hosts (Suttle et al. 1990; Proctor and Fuhrman 1992; Suttle 1992; Peduzzi and Weinbauer 1993; Weinbauer and Peduzzi 1995; Noble et al. 1999). The difference, here, is that we are applying Landry's dilution method using diluent prepared with ultrafilters that have 10X smaller porosity than used previously, such that the water is virus free. This allows us to derive rates of production of CDOM associated with particles in the virus size range (e.g. viruses). Moreover, inclusion of a raw seawater sample without augmented viruses, allows us to compare any virus effect on CDOM production with the cumulative effect of all other processes, including effects of bacteria and zooplankton. Time courses of absorption, scattering, fluorescence, particle size distribution and virus concentration were performed. The simplicity of this experimental design is that we can directly target the role of viral lysis in the production of CDOM. Note, the dilution experiments can also change the concentration of polymer gels (by adding 0.02μm-filtered, virus-free seawater as diluent). Therefore, we will perform dilution and aggregation experiments in the same water samples so that we will have the necessary controls to correct for any aggregation effects during the virus dilution experiments.

Aggregation work- We also are examining the optical properties of aggregates, controlling for microbial activity and aggregate growth and testing for non-biogenic calcite formation associated with aggregates, under controlled conditions. Microaggregates have been hypothesized to be a potential source of CaCO3 (Chin et al. 1998). The experimental design involves ultrafiltering natural seawater samples through 0.02μm filters (to remove all viruses, cells and large colloids) and then examining formation of aggregates and calcite in the filtrate over time as small polymers anneal. Analyses of particulate calcium (using inductively-coupled plasma atomic absorption), particle size (flow-field flow fractionation) and parallel measurements of optical volume scattering and absorption are performed. Controls include treatments with EDTA (to inhibit aggregate formation) and sodium azide (to provide for biological versus abiological changes). By combining the scattering observations with aggregate concentration, we can calculate the scattering cross-sections of aggregates, essential for estimating their optical impact in the sea.

WORK COMPLETED (JANUARY TO SEPTEMBER '05)

Virus isolation- During the first portion of this project, we isolated, characterized, purified and enumerated viruses infecting naturally-occurring heterotrophic bacteria using techniques of Balch et al. (2000), with samples taken during cruises to the Gulf of Maine. We adapted these methods for use

with the coccolithophore, *E. huxleyi* (clone 88E*)*. We also completed primary characterization, morphological analysis, and studies of growth kinetics and infectivity parameters of three of the *E. huxleyi* virus isolates (ø 28, 29, 30). Three enumeration methods were adapted for use with *E. huxleyi* viruses. We used our previously published methods for the production of highly purified virus concentrates (Balch et. al, 2000). Electron microscopy was performed with a Philips EM 201 transmission electron microscope.

Virus growth kinetics were assessed using a single-step growth experiment. Exponentially-growing *E.huxleyi* 88E host cells were concentrated via centrifugation and resuspended in a 25-ml volume of f/2 medium at a final concentration of 4.3e6/ml. Host concentrates were then either inoculated with sufficient virus stock to give a multiplicity of infection (MOI) of 1.0, or left uninoculated (controls). Studies were also conducted to determine the effects of dilution (decreasing MOI and reduced virus/host concentrations) in natural seawater on virus infectivity and utilized purified stocks of one of the isolated *E. huxleyi* viruses (ø 28). We used ambient seawater, rather than growth medium, to better replicate the prevailing conditions (chemical, nutrient, etc) existing in the Gulf of Maine. Prior to the experiments, seawater was ultrafiltered to remove viruses. For MOI experiments, virus-free seawater was inoculated with sufficient exponentially-growing *E.huxleyi* 88E. Experimental flasks were then inoculated with ten-fold dilutions of ø 28 to yield MOI's ranging from 1.0 (i.e. 1 virus /host cell) to 0.00001. Flasks (including virus-free host controls) were incubated with daily observations of host and virus concentrations. In other experiments, virus-free seawater was inoculated with host/virus concentrations ranging from 1e5/ml to 1e1/ml, with the MOI kept constant at 1.0. Flasks were incubated for 7 days, with daily readings of host and virus numbers. For flasks containing very low initial host/virus concentrations (i.e. 1e1/ml and 1e2/ml), it was necessary to concentrate host by centrifugation prior to counting.

Dilution experiments with known host-virus combinations- Dilution-infection experiments were performed on heterotrophic bacteria (ø K-4 and host *Photobacterium angustum* or K-5 and host *Vibrio harveyi*) as well as the coccolithophore, *E. huxleyi* (ø 28). Host cells were split 1:2 into virus free GOM water and incubated for 4d for host acclimation. Next, host cells were pelleted and resuspended in virus free seawater. Host and virus (MOI=1) were then added to flasks containing virus free seawater at the following host/virus concentrations: 100% (~e5/ml), 70%, 50%, 30% and 5%. Control flasks (only host, virus or seawater) were also prepared. Flasks were assayed daily for changes in host and virus concentrations. Optical measurements involved spectral measurements of chromophoric dissolved organic matter (CDOM) over time in each of the dilutions and controls. Samples were initially prefiltered through 0.2um so that they represented the generic size range of dissolved organic matter (all matter passing a 0.2μm filter). Volume scattering and fluorescence measurements were also made to assess the viral impact on elastic and inelastic scattering.

Aggregation Experiments- Controlled aggregation experiments were performed, starting with sterile cultures of E. huxleyi, which were concentrated over 0.2um filters. The retentate was centrifuged to pellet particulate matter. The pellet was resuspended, sonicated to break cells and release DOC, then filtered through 0.2 μm filters. This DOC was then added to 0.02μm filtered seawater and a time course of spectral absorption and volume scattering measurements performed (using both unfiltered and 0.2μm-filtered material). The distribution of nano- to micron- sized particles over time was investigated using a Flow Field Flow Fractionator (FFFF; Postnova Analytics) equipped with two high precision pumps for maintaining the rates of the channel and cross flow during particle separation. A regenerated cellulose membrane of nominal molecular weight cutoff of 10,000 Daltons served as the

semi-permeable membrane within the channel. An Alltech UV absorbance detector was used to detect particles. 0.02 μm filtered seawater from the Jordan Basin, Gulf of Maine containing a non-ionic surfactant (final concentration 0.1% v/v) served as the carrier solution and minimized interactions between individual particles and reduced particle adhesion to the semi-permeable membrane. Mono-disperse suspensions of polystyrene latex beads (19 to 350 nm) were used to establish a standard curve, which provided an elution time-dependent measure of particle.

RESULTS

Virus Infection Experiments- Eight clonal isolates of *E. huxleyi* 88E viruses have been recovered from GOM waters for use in lab-based dilution experiments. Characterizations of 3 of the isolates have revealed double-stranded DNA containing icosohedral particles ranging in size from 130 to 160 nm (Fig. 1). None have been shown to possess a lipid envelope. These viruses are extremely stable in seawater over a wide range of temperatures (-72C to 17C). They produce well-defined plaques in host lawns (Fig. 2). Single step growth studies have showed most virus progeny being produced during the first 24h post-infection, a period when there is little observed change in corresponding host numbers (which do not radically change until >50 hr post infection). This finding suggests that viruses "leak" out of still viable infected host for a considerable period prior to eventual lysis of the host. The average number of virus particles produced per infected cell appears to be ~1000. Results of viral assays from fresh lysates (MPN, plaque counts) compare favorably with SYBR green virus particle counts. This suggests a high virus infectivity level (i.e. most progeny viruses are infective) not previously demonstrated for *E. huxleyi* viruses. Infectivity of the viruses is not significantly diminished when the virus/host ratio (multiplicity of infection-MOI) is reduced to 0.00001 virus particle per host cell. Dilution of virus/host concentrations (MOI held constant at 1.0) to ~100/ml does not significantly effect virus infectivity (i.e. their ability to locate and destroy their host). This has implications for our dilution experiments where virus/host concentrations are >5000/ml, even at the lowest dilutions used.

Dilution-infection experiments demonstrated clear affects of dilution on propagation rates in virus concentration and mortality rates of hosts. Indeed, viral infection is rapid; propagation rates of viruses and mortality rates of hosts (over first 20h of experiment) are a function of dilution below the 70% dilution flask (Fig. 3). That is, from 5% to 70% dilutions, there is a roughly linear increase in viral propagation rates and host mortality rates. Moreover, controls without viruses showed host mortality rates of zero. As expected, controls with viruses and no hosts showed a propagation rate of zero. Interestingly, there is a positive Y intercept in Fig. 3 for the viral propagation rates. Since we know from the control that the viral growth rate is zero in the absence of host (equivalent to a dilution of 0% (infinite dilution) then between 5% dilution and 0%, the viral propagation rate must show a marked decrease. When Bigelow Laboratory dock water was used as a diluent instead of 0.02μm filtered water, the host mortality rate was almost the same, indicating that the infection was not affected by other, naturally-occurring particles. There was clear indication of CDOM release as a function of infection in the dilution experiments (Fig. 4). It can be seen that the rate of increase of absorbance (h^{-1}) was strongly correlated with the dilution. This plot had a significant Y intercept, indicative of the natural CDOM release rate of the hosts (which was identical to the control host CDOM release rate,with no added viruses). As expected, the CDOM release rate in the ultra-filtered sea water control was negligible, and lowest of all treatments.

Aggregation experiments- FFFF fractograms obtained at time-0, after filtration of concentrated DOM through a sterile 0.2μm filter, revealed the presence of particles within two size ranges, 10 to 30nm and

50 to 250 nm (Fig. 5a). The DOM concentrate was added to each of the experimental flasks containing 0.02μm filtered seawater and treated either with EDTA, sodium azide or no treatment at all. FFFF measurements revealed particles whose size distribution clearly mimicked that of the concentrate (50 to 250 nm and 10 to 30nm) (Figs 5b-d). The intensity of the peaks however, was clearly lower than that of the DOM concentrate, obviously due to the dilution. FFFF measurements on samples drawn from DOM concentrate and from the treatments on Days 1, 3 and 4 (no sample was collected from DOM concentrate on Day 4), revealed changes in particle sizes that differed markedly between treatments. For example, the DOM concentrate revealed a shrinkage in size of the peak between 50 and 300nm and the formation of particles that were > 1μm over time (Fig. 5a). For untreated DOM, the prominent peak at about 125nm noted on Day 0, was replaced by a larger peak about 225nm by Day 3, clearly a sign of polymer gel aggregation and the formation of larger particles (Fig. 5b). By Day 4, no peaks > 100nm could be detected in the sample. Microscopic examination revealed that these flasks were contaminated with rod shaped bacterial cells, suggesting possible bacterial breakdown of the larger, 50 to 250 nm sized particles, to form smaller particles between 10 and 30nm and larger particles that were > 1μm. The idea that bacterial breakdown could have been responsible for the disappearance of the larger (50-250nm) particles and the prevention of aggregation, is strengthened by the observations within the sodium azide-treated flasks where a slow, but clear, increase in >200nm particles was seen, even on Day 4 of the experiment (Fig. 5c). Aggregation of particles within the sodium azide-treated flasks also suggests that, apart from biogenic processes, non-biogenic processes play an important role in aggregate formation. For EDTA-treated DOM, there was an apparent breakdown of particles between 100 and 150nm to form smaller, 10-30nm particles (Fig. 5d). Even time-zero samples showed enhanced particles <=10nm. By Day 1 and continuing into Days 3 and 4, there was a clear reduction in the prominent 125nm peak (noted on Day 0) and an increase in the particles between 10 and 30nm. Beyond 150nm, FFFF fractograms revealed no apparent change in particle size distribution even on Day 4 of the experiment. The disintegration of large particles to form smaller particles in these flasks supports the observation that EDTA prevents polymer gel aggregation as was reported previously by Chin et al. (1998). But it may be noted that the presence of EDTA had no effect on particles greater than 150nm in our experiments. Backscattering within the DOM-only flask showed dramatic increases in bb at 531nm, due from both polymer gel aggregation as well as bacterial contamination (Fig.6). EDTA-treated DOM showed 5X the backscattering of the sodium-azide-treated samples beginning at zero-time, presumably due to the prevalence of smaller particles (Fig. 4). Backscattering of the sodium azide-treated DOM gradually increased, illustrating non-biogenic effects of aggregation. The 0.02um-filtered seawater maintained the lowest backscattering of all, yet still with some measurable increases as particles formed from the small amounts of DOM contained within it.

IMPACT/APPLICATIONS

This work will have impact in two areas: a) understanding the impact of viral infection on the optical properties of marine bacteria and phytoplankton (particularly, the magnitude of changes in inherent optical properties, rates of change and host specificity), plus b) optical properties associated with polymer-gel formation. Given that polymer gels are one of the most abundant particles in the sea, the results of this work could have far-reaching impact on our understanding of ocean optical properties, particularly light scattering.

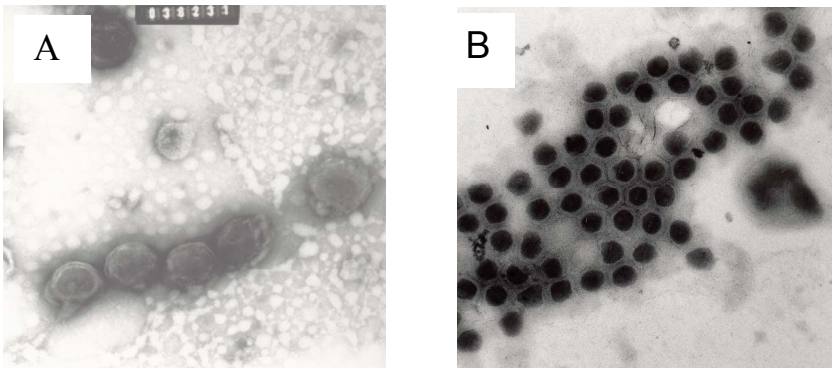

Figure 1.Scanning electron micrographs of E. huxleyi virus isolates. A) Virus Isolate ø 28. B) Virus Isolate ø 30.

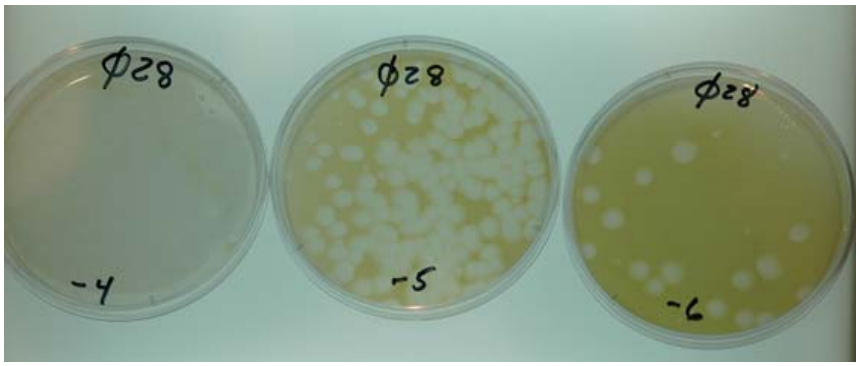

Fig. 2- Virus Isolate Plaques on E. huxleyi Host Lawns. Viruses were layered on host plates at concentrations of 10^{-4} ml^{-1}(left), 10^{-5} ml^{-1} (middle), 10^{-6} ml^{-1} (right). Plaques can clearly be seen, especially at the lower two concentrations.

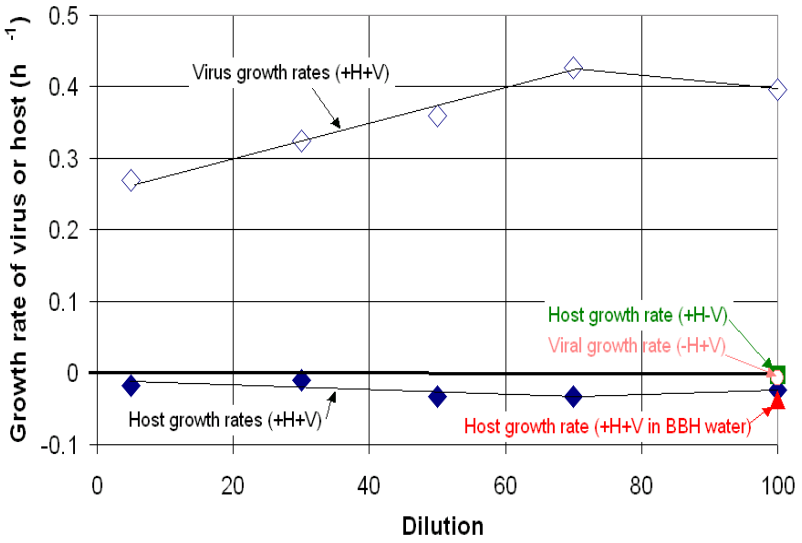

Figure 3- Results of infection dilution experiment showing specific growth rates of viruses and hosts as function of dilution. Growth rates were measured over first 20h of the experiment. Note, a negative growth rate represents mortality via lysis. "+H" or "–H"means host present or absent. "+V" or "–V" means virus present or absent. Open symbols are virus growth rates while closed symbols are for E. huxleyi growth rates. Open diamonds and solid diamonds represent virus and host growth rates, respectively in infected flasks at different dilutions, all at an MOI of 1.

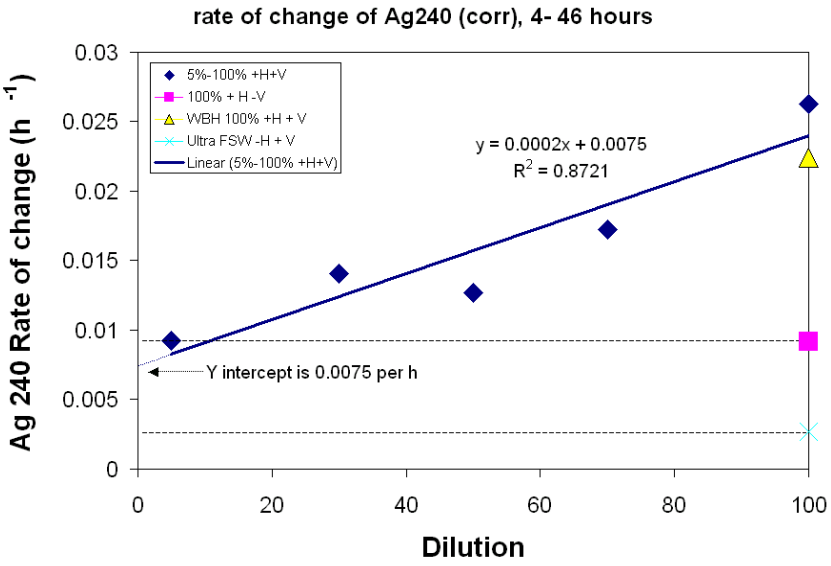

Fig. 4-Results of dilution experiment with Emiliania huxleyi (strain 88E) and its virus. In this experiment, infection rate is controlled by the dilution of the host with 0.02um-filtered water. CDOM production is measured as the rate of change of absorbance at 240nm. The figure shows how the rate of release of CDOM slows as the samples are diluted in concentration from 100% to 5% of the original concentration (blue squares). The Y intercept represents the natural release of CDOM with no viruses present (infinite dilution). The pink square denotes the 100% dilution for hosts, and no viruses present. The dashed line connected to the Y axis shows that this value is close to Ag240 expected for infinite dilution (no virus infection, only natural leakage from host cells. The yellow triangle shows the absorbance change expected for a 100% host and virus concentration but the host and virus were added to raw seawater off the Bigelow Laboratory dock, not ultra filtered water. Note, the rate of change of the Ag240 was about the same as if the sample had been diluted with 0.02um-filtered water. The turquoise X denotes ultra-filtered seawater with neither virus nor host and demonstrates the CDOM in the ultra-filtered water, unrelated to host or virus concentration.

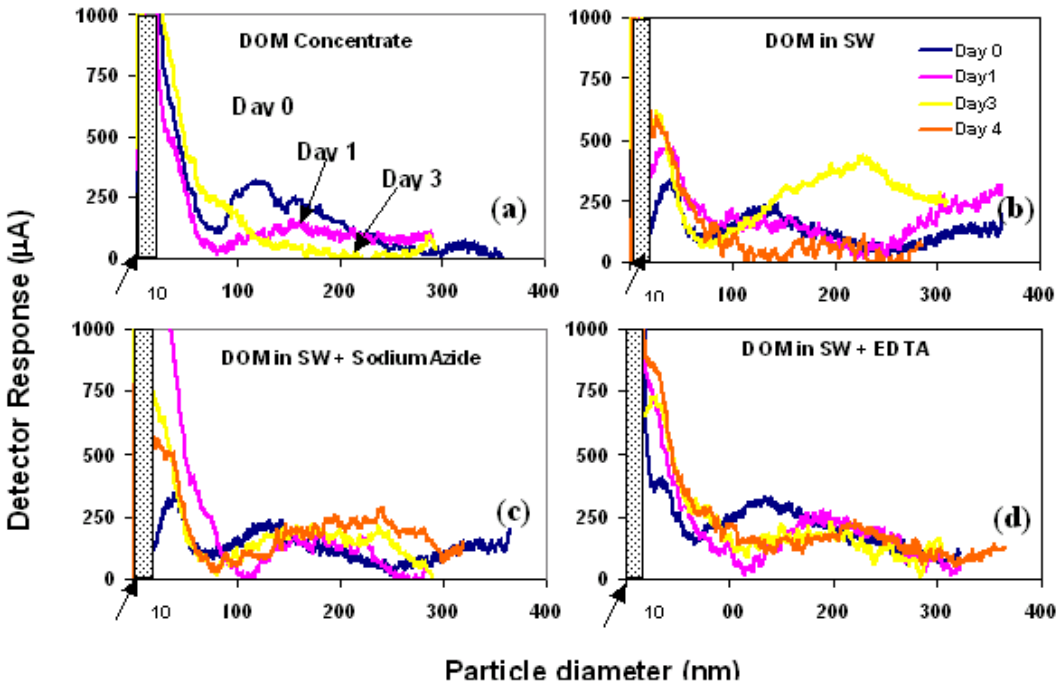

Fig. 5. Flow Field-Flow Fractograms of samples from aggregate experiments over time a) DOM concentrate b) DOM in seawater c) DOM in seawater with sodium azide d) DOM in seawater with EDTA. Hatched area to the left of each panel of fractograms (designated by arrows) represents particles greater than 1µm. The reason for this is that for such particles, the mechanism of particle migration through the channel undergoes a transition from the normal to the steric mode. In this mode, larger particles (with negligible diffusion), when driven forcefully by cross-flow towards the accumulation wall, are lifted back towards the long axis of the channel by hydrodynamic forces and carried out of the channel faster than the smaller particles.

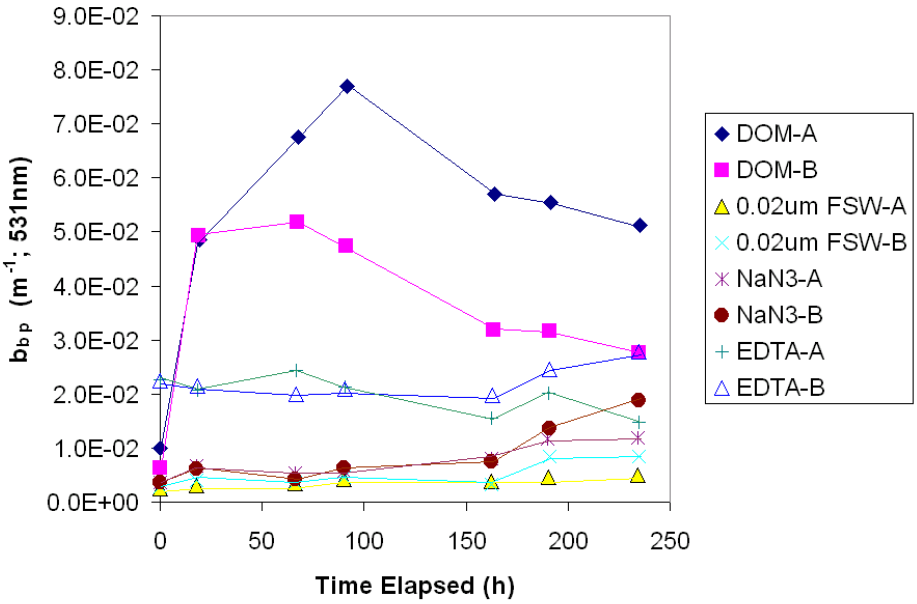

Fig. 6- Time course of particle backscattering in flask which originallys contained 0.2um-filtered DOM at time zero. Legend designates replicate treatments "A" and "B" (◆■--CDOM only, HX -- 0.02um filtered seawater,X J --DOM + sodium azide,+ Δ --DOM+EDTA). Note rapid increase in backscattering inDOM-only flask, then decrease after 96h. Also note enhanced backscattering in EDTA-treated flasks (the stock EDTA solution did not have as much backscattering). Backscattering in sodium azide-treated DOM gradually increased, representing non-biogenic effects of aggregation. 0.02um filtered seawater consistently showed the lowest backscattering. --

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